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Fine Structure of the Iris Muscle in the Japanese Common Newt, *Cynops pyrrhogaster*, with Special Reference to Innervation

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ABSTRACT—The localization and structure of the iridial muscles and associated nerves of the newt (*Cynops pyrrhogaster*) were examined by electron microscopy with some histochemical studies of catecholamine and acetylcholinesterase. The sphincter muscle, which was composed of pigmented smooth muscle cells, was located circumferentially in the pupillary margin of the iris. Sphincter muscle cells formed occasional close contacts with each other by protruding cellular processes. Numerous gap junctions and desmosomes were observed between anterior and posterior pigment epithelium of the iris, but not between muscle cells. Nerve endings formed varicosities and were composed of agranular vesicles and/or granular vesicles. There was no dilator muscle in this species. Prominent circumferential catecholamine fluorescence was observed in the pupillary margin. Acetylcholinesterase positive and negative muscle cells and nerve fibers were found within the sphincter muscle region.

INTRODUCTION

The iris sphincter and dilator muscles, known to be the iris muscle in the mammalian eye, change the size of the pupil and regulate the quantity of light. Studies on the iris muscles have been a fascinating subject from the standpoint of development and differentiation of the muscle, because these muscles are unique in that the sphincter muscle is the only vertebrate smooth muscle known to be derived from neuroectoderm [1–4] and the dilator muscle has myoepithelial characters [1, 5–6]. They are innervated by an autonomic nervous system [7–10], which is advantageous, in that we can easily detect the action of nerves through miosis and mydriasis of the eye. In addition to morpholgical studies on the iris muscle [1–6, 11–13], numerous pharmacological and electrophysiological reports have been made on this nervous system using various kinds of animals [14–20]. Studies on the fine structure of the iris muscle besides mammals have also been reported [21–24], but only a few works have been undertaken on amphibian species [25–27].

On the other hand, it has been well known that the newt has a capacity for regeneration from the mid-dorsal margin of the iris after having extirpated an intrinsic lens. Numerous investigations at the light and electron microscopic level have been made on the process of lens regeneration (for review, see [28]). However, the iris muscle in the newt has so far received almost no attention in terms of the study of lens regeneration, although the sphincter muscle at the pupillary margin would appear to be closely relevant to lens regeneration.

There have been no reports on the fine structure of the iris muscle of the Japanese common newt, *Cynops pyrrhogaster* and no histochemical works on amphibian iridial muscles and nerves. Thus, in the present study, detailed investigations on the fine structure of the iris muscle and some histochemical studies on the iridial muscles and associated nerves were made in the adult *Cynops pyrrhogaster*. In addition, the present study also seeks to obtain basic information on the iris muscle, which presumably has some relevance to lens regeneration.

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MATERIALS AND METHODS

Electron microscopy

Adult Japanese newts, *Cynops pyrrhogaster,* were kept in an aquarium at 21±2°C. Newts for experiments were decapitated and the isolated dorsal heads were prefixed at 4°C overnight in 3–6% glutaraldehyde in Hanks' solution diluted to 80% of the original concentration for newts. After rinsing with the buffer, iridocorneal complexes were isolated and postfixed in cold 1% osmium tetroxide in the buffer for 1 hr. The tissue fragments were then block stained with 0.5–1% aqueous uranyl acetate solution for 1 hr, washed, dehydrated in graded series of ethanol, and embedded in Epon. Sections were cut with a glass knife or a diamond knife with a Reichert ultramicrotome, collected on carbon-coated grids, stained with uranyl acetate and lead citrate, then examined by a JEOL 100C electron microscope at 80 KV.

Histochemical localization of catecholamines

The glyoxylic acid fluorescence technique [29] was employed with a slight modification for the newt iris. The isolated iris rings of the newts were immersed in 2% glyoxylic acid in 0.1–0.2 M Sörensen's phosphate buffer (pH 7.0) for 30 min. Samples were mounted on slides and air dried, then heated for 4 min on a hot plate at 100°C. Control samples were treated only with the buffer. The specimens were sealed in liquid paraffin and observed with fluorescence microscope.

Demonstration of acetylcholinesterase at the electron microscope level

For the demonstration of sites of acetylcholinesterase activity, the method of Karnovsky and Roots [30] modified by Tsuji [31] was employed with a slight modification for the newt iris. The specimens were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.05–0.1 M Sörensen's phosphate buffer (pH 7.4) at 4°C for 2–3 hr and washed overnight in the buffer. After rinsing in 0.1 M acetate buffer (pH 6.2), the samples were placed in the incubation medium (1.7–8.5 mM acetylthicholine iodide, 0.1 M sodium acetate, pH 6.2, 0.01 M copper sulfate, 0.04 M glycine, and 0.04 M magnesium chloride) for 3 hr at room temperature. Thereafter, the tissues were rinsed twice in the acetate buffer and placed in 3% potasssium ferricyanide solution for 30 min. All incubations were performed in the dark with gyration. After a rinse in the phosphate buffer, the tissues were processed for electron microscopy as previously described. Eserine (physostigmine), an inhibitor of cholinesterases, was used at the concentration of 10^{-4} M to detect the presence of nonspecific esterases. Tissue samples were preincubated for 15 min in an eserine solution, and placed in the incubation mixture containing an inhibitor at the same concentration.

RESULTS

General features and localization of the iris muscle

The iris can be easily recognized as a three-

Fig. 1. Meridional section of the iris. The stroma of the iris faces the cornea. Montage. Bar represents 20 μm.

Fig. 2. Junctions between anterior and posterior pigment epithelium. Both a gap junction and desmosomes are present side by side. ×23,000.

Fig. 3. A longitudinal section of the pigment epithelium. The surface of the pigment epithelium is surrounded by a basal lamina. ×23,000.

Fig. 4. A higher magnification of the sphincter muscle region shown in Fig. 1. The region is prominent with a scarcity of pigment granules within the cell. ×2,400.

Fig. 5. A horizontal section cut slightly obliquely to the iris diaphragm. The sphincter muscle cells are located parallel to each other and to the pupillary margin. ×1,300.

Figs. 1–16 are all electron micrographs. Abbreviations: st, stroma; ap, anterior pigment epithelium of the iris; pp, posterior pigment epithelium of the iris; sm, sphincter muscle; g, gap junction; d, desmosome; bl, basal lamina; Nu, nucleus; mf, myofilament; db, dense body; pr, polyribosome; fr, free ribosome; m, mitochondria; pg, pigment granule; ne, nerve ending; J, cell junction; col, collagen fiber; agv, agranular synaptic vesicle; gv, granular synaptic vesicle.

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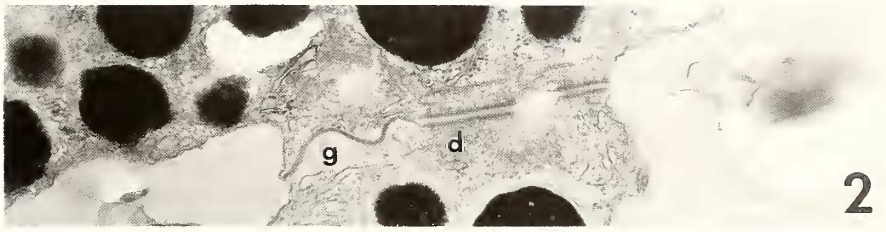

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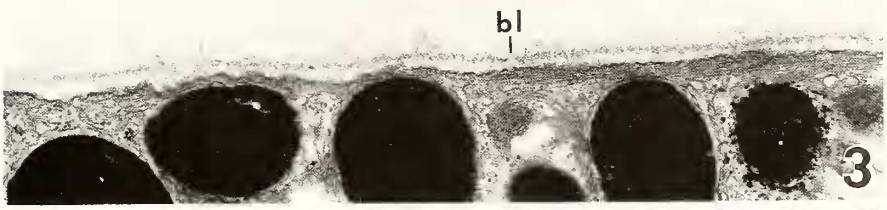

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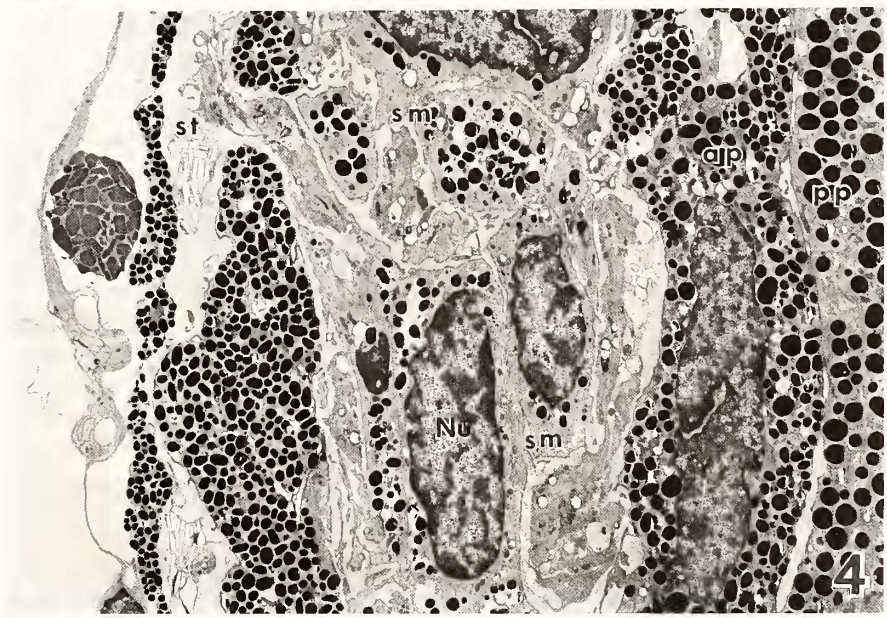

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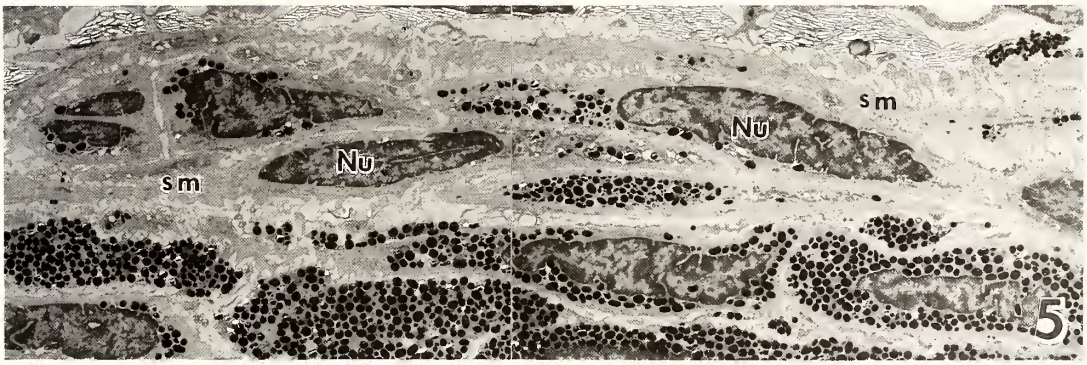

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layered structure (Fig. 1). The most anterior layer is the stroma of an iris, which contains several cell types of mesenchymal cells and blood vessels. The iridial stroma is rather scanty compared with the other two layers. The next layer is the anterior pigment epithelium. It is laterally continuous with the pigment epithelium of the retina. It comprises a single layer of cells filled with melanin granules which are round to oval in profile, and about $0.6\times0.8\,\mu$m in diameter. The third layer is the posterior pigment epithelium. It is laterally continuous with the neural retina, also comprising a single layer of cells filled with round, relatively large melanin granules, about 1 μm in diameter. Numerous gap junctions and desmosomes can be found between the anterior and posterior pigment epithelium (Fig. 2). The anterior and posterior surfaces of the pigment epithelium are surrounded completely by a basal lamina (Fig. 3). The sphincter muscle cells are between the stroma and the anterior pigment epithelium (Fig. 4). They enclose a pupillary margin like a ring and are located parallel to each other and to the pupillary edge (Fig. 5). As the sphincter muscle cells have more scanty pigment granules than the surrounding pigment epithelial cells, their region can be easily recognized in the low magnification electronmicrograph (Figs. 1, 4 and 5).

Fine structure of iris muscle cells and associated nerve fibers

The individual sphincter muscle cells contain bundles of myofilaments along the long axis of the cell (Figs. 6 and 7). In cross section of the filament bundles, thin (about 7 nm in diameter) and thick (about 20 nm in diameter) myofilaments can readily be identified (Fig. 8). Dense bodies or dense plaques were scattered throughout the bundles of myofilaments and on the cytoplasmic side of the plasmalemma (Fig. 6). Nuclei were slender in outline with many interdigitations along the nuclear envelope. Mitochondria, free ribosomes and polyribosomes were seen in the vicinity of the conglomerizations of pigment granules and beneath the cell periphery. Average diameter of the pigment granules in the sphincter muscle cells was $0.5\times0.7\,\mu$m; their size was consistent with those of the anterior pigment epithelium.

Many smooth-surfaced vesicles were interspersed along the plasmalemma (Figs. 6, 8 and 9). These vesicles have been called caveolae, plasmalemmal vesicles, or micropinocytotic vesicles in the cells of the smooth muscle. In Figure 9, we can easily identify the two different figures of caveolae. In sections tangential to the cell surface, the caveolae were seen in circles, but in vertical sections, they were seen in a flask-shaped invagination attached by a narrow neck region. Each sphincter muscle cell was surrounded by a basal lamina except the spots at which the muscle cells were closely adjacent (Figs. 6 and 10). The sphincter muscle cells usually formed close contacts with neighboring cells by protruding cytoplasmic processes. But no gap junctions and desmosomes were seen between muscle cells, in contrast to the junctions found between the anterior and posterior pigment epithelium. Collagen fiber or bundles of fibers were occasionally present in the

FIG. 6. A longitudinal section of the sphincter muscle cell. Myofilaments, dense body, pigment granules, caveolae, free and poly ribosomes are found within the cytoplasm. Each muscle cell is in close contacts with cellular processes. ×13,500.

FIG. 7. A higher magnification of the myofilament bundle sectioned longitudinally to the bundle. Each myofilament is clearly discernible. ×37,000.

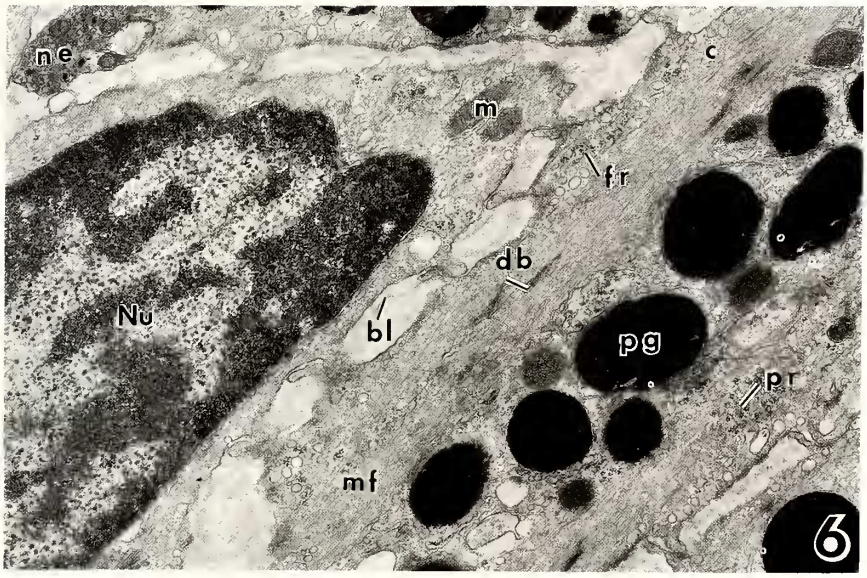
FIG. 8. A higher magnification of the myofilament bundle sectioned crossly to the bundle. Thick (large arrow) and thin (small arrow) myofilaments are prominent. Numerous caveolae are also found at the cell periphery. ×37,000.

FIG. 9. Section cut partly tangential and partly longitudinal to the cell surface, showing crossly and longitudinally-sectioned caveolae, respectively. ×19,800.

FIG. 10. The junction between two sphincter muscle cells. They closely contact each other with protruded cellular processes. ×18,150.

FIG. 11. Collagen fiber or bundles of fibers scattering in the intercellular spaces of the sphincter muscles. ×66,300.

FIG. 12. Meridional section of the iris situated between the sphincter muscle region and the root of the iris. There are no dilator muscles on the anterior pigment epithelium facing the iridial stroma. ×3,960.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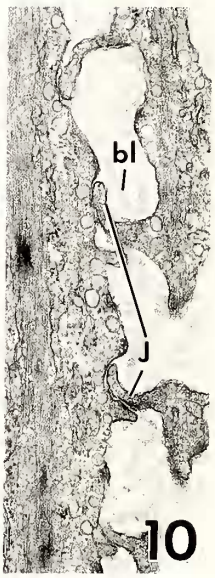
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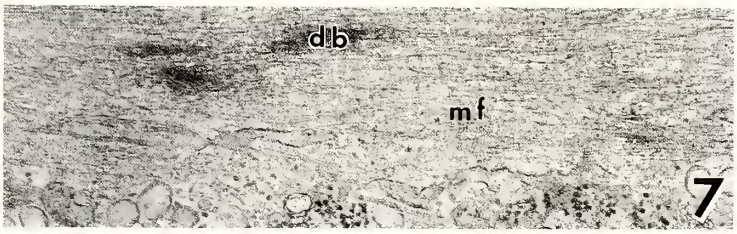
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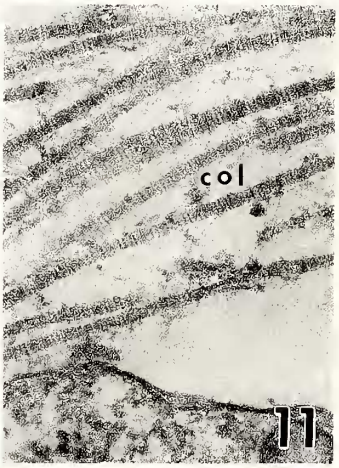
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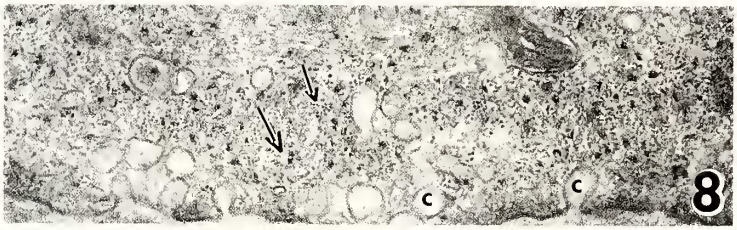
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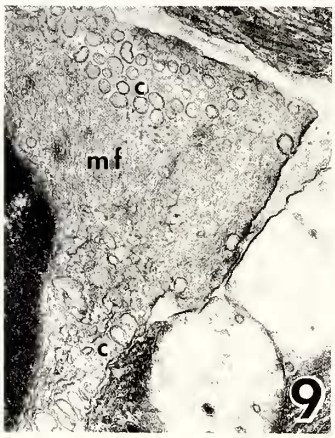
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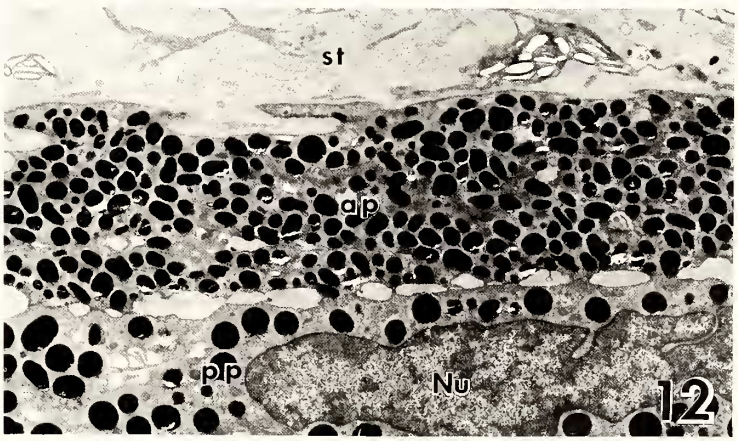
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intercellular spaces (Fig. 11).

Mammalian dilator muscle is knowm to be situated peripheral to the sphincter muscle and to be continuous with the cell bodies of the anterior pigment epithelium [32]. The dilator muscle is therefore a partial specialization of cytoplasmic processes of the anterior pigment epithelium into the myoepithelium. The dilator processes in mammals are arranged in an overlapping manner somewhat like tiles on a roof. In the iris of *Cynops pyrrhogaster*, the dilator muscle could not be found even in detailed observations in the corresponding region of the mammalian dilator muscle (Fig. 12).

Large bundles of nerve fibers locating near the muscle cells were found at various places in the sphincter muscle region (Fig. 13). In cross and longitudinal section of nerve fibers, microtubules and neurofilaments were seen along the long axis of the fibers. Nerve endings were found close to the muscle cells (Fig. 14). A cleft of about 5–10 nm between nerve membrane and muscle membrane was usually found, but in most examples, the area of contact between nerve endings and muscle cells did not show junctional specializations. Nerve endings which usually form varicosities contain agranular (40–60 nm in diameter) and/or granular (90–130 nm in diameter) synaptic vesicles (Figs. 15 and 16). Varicosities were roughly classified into two types. One is composed mostly of agranular vesicles of rather uniform diameter except the occasional existence of only few granular vesicles. Another is composed of granular, dense-cored vesicles and agranular, clear vesicles.

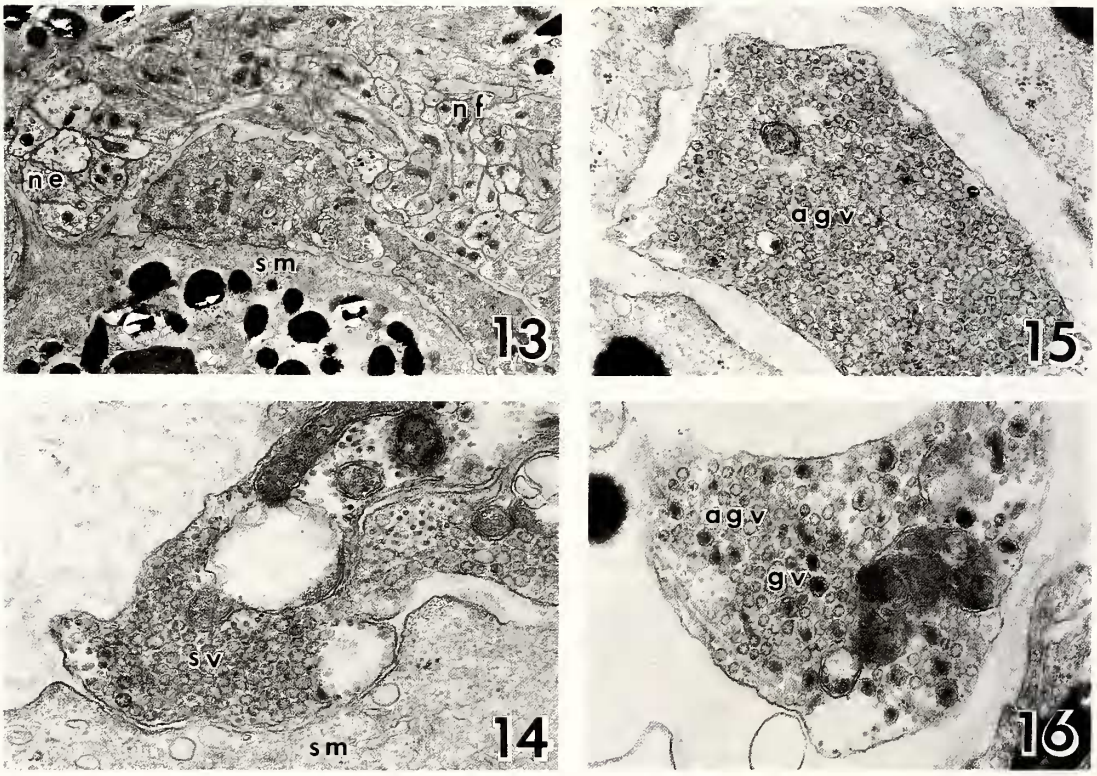

Fig. 13. Section showing dense innervation in the sphincter muscle region. ×6,435.

Fig. 14. Adjacent contact of a nerve ending and a sphincter muscle cell. The nerve varicosity contains numerous agranular synaptic vesicles. ×29,500.

Fig. 15. A nerve varicosity of agranular type. Almost all of the synaptic vesicles are composed of agranular vesicles. ×25,000.

Fig. 16 A nerve varicosity of granular type. Agranular and granular vesicles are mixed in a varicosity. ×25,000.

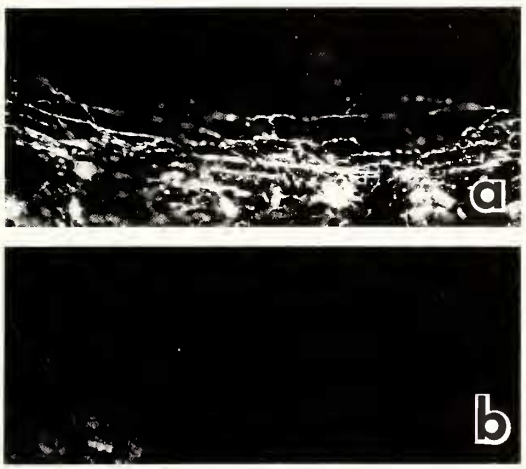

FIG. 17. Histochemistry of catecholamine in the pupillary margin. a, Fibrous catecholamine fluorescence is found circumferentially at the pupillary margin. b, No fluorescence at the pupillary margin is found in the buffer-treated sample. ×178.

Localization of catecholamine and demonstration of acetylcholinesterase activities in iris muscle

The fluorescence micrographs of pupillary margin of the iris treated with glyoxylic acid are shown in Figure 17a. Fluorescence was prominent in fibrous structure circumferentially located at the pupillary margin. The fibers showed a knot-like structure in some places. No fluorescence was detectable in the buffer-treated samples (Fig. 17b). In samples treated with acetylthiocholine as the substrate of acetylcholinesterase, the enzymatic reaction product was observed randomly in the surface of the sphincter muscle cells (Fig. 18a). The precipitate also appeared randomly at nerve fibers and vesicle-filled varicosities. In addition to the presence of the acetylcholinesterase-positive cells and nerve fibers, there were some negative cells and nerve fibers associated with the surrounding muscle cells (Fig. 18b). They appeared to have the same features as eserine-treated samples (Fig. 18c).

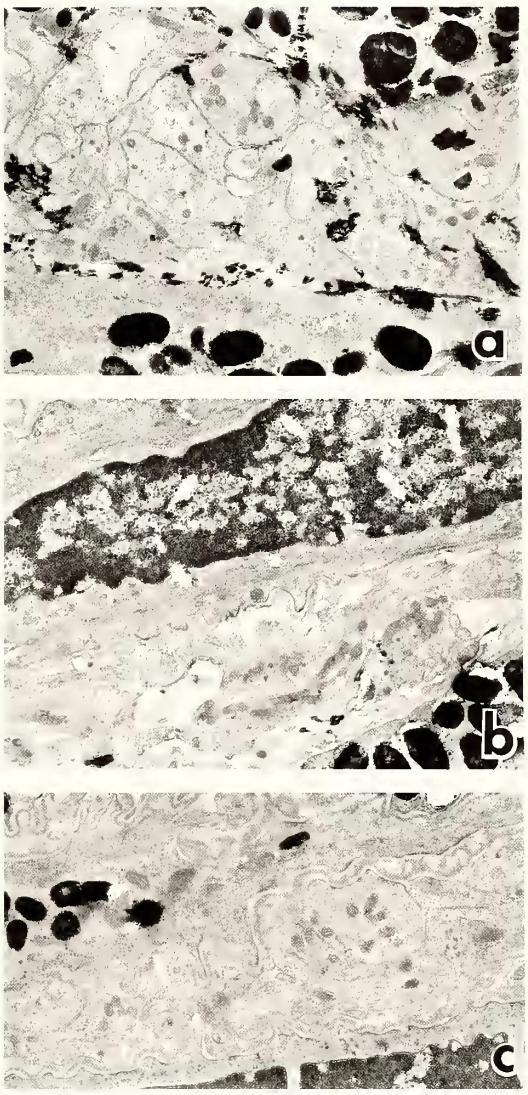

FIG. 18. Histochemistry of acetylcholinesterase in the sphincter muscle region. a, Acetylcholinesterase-positive muscles and nerve fibers are present in the samples treated with acetylthiocholine as the substrate. b, Acetylcholinesterase-negative muscles and nerve fibers are also found in the sample treated with the substrate. c, No reaction product is found in the eserine-treated samples. ×7,750.

DISCUSSION

Present study firstly demonstrated the localization and the detailed structure of the iris muscle in the Japanese common newt, *Cynops pyrrhogaster*. In addition, some histochemical studies on the localization of catecholamines and acetylcholinesterases were made in the iris muscle of the newt. The sphincter muscle is usually classified as the smooth muscle, even if its developmental

origin is very different from ordinary smooth muscle of mesodermal origin [32]. In contrast to the mammalian sphincter muscle, the iris muscle in the newt appears to maintain some characters of the pigment epithelium of the iris by including a considerable number of cytoplasmic pigment granules within the muscle cells even in adult age. The persistence of a considerable number of pigment granules within the muscle cells and the lack of the dilator muscle in *Cynops pyrrhogaster* are consistent with results in other amphibian species [25–27]. These results suggest an incomplete differentiation of the iris muscle in the amphibian eye.

The present results showed that the sizes of the pigment granules in the sphincter muscle cells were similar to those of the anterior pigment epithelium. But, in the American newt, *Taricha torosa,* the sizes of the pigment granules were similar to those of the posterior pigment epithelium [26]. Based on this observation, Tonosaki and Kelly [26] proposed the notion that the sphincter muscle was derived from the posterior pigment epithelium. On the other hand, in the grass frog, *Rana pipiens,* the pigment granules of the muscle cells were of an intermediate size of the anterior and the posterior pigment epithelium [27]. Thus, it seems not to be adequate to amplify the notion about the origin of the sphincter muscle obtained in *Taricha torosa* to all amphibian species.

It has been generally accepted in the mammalian eye that the iris sphincter or dilator muscles are innervated by excitatory cholinergic or adrenergic nerve fibers, respectively, and miosis or mydriasis is the result of contraction of these nerve fibers [33]. Then the question arises as to how miosis or mydriasis is caused in the newt eye which has no dilator muscle. Some suggestions will be provided in the present results and in those previously reported by others [12, 25]. Two types of varicosities were observed in nerve endings in the present study. Richardson [12] has described two types of nerve endings in the iris muscle of the rabbit, the first containing numerous small, agranular vesicles with an occasional large dense-cored vesicle, the second also with agranular vesicles, but mixed with a large number of dense-cored vesicles of two different types. He suggests that the first, associated with the sphincter, may be typical of cholinergic innervation; the second, associated with the dilator, typical of adrenergic innervation. The features of nerve endings found in the iris muscle of the rabbit were very similar to those in *Cynops pyrrhogaster.* However, in the *Cynops,* there is no dilator muscle.

Armstrong and Bell [25] found that the toad possesses no dilator muscle and the application of noradrenaline or sympathetic nerve stimulation causes pupillary dilation and acetylcholine or parasympathetic nerve stimulation produced pupillary constriction. From these results, they concluded that the sphincter muscle in the toad has a dual innervation. Based on the pharmacological and electrophysiological works, a dual innervation of the mammalian sphincter and dilator muscle has been postulated in various species including cat, rat, bovine, dog and human [14–20]. The present results may be explained by dual innervation of the sphincter muscle as in the toad. But it is also probable to postulate that both the sphincter and the dilator muscle are situated so to be mixed in the so-called mammalian "sphincter region." The existence of acetylcholinesterase-positive and -negative muscle cells and nerve fibers in the "sphincter region" may support this idea. Because a dual innervation in only one type of the muscle makes it difficult to explain the mixed existence of acetylcholinesterase-positive and -negative muscles. Further examinations of the newt iris muscle will provide additional data to the above two possibilities. However, it is said that acetylcholinesterase is not always an appropriate marker for the cholinergic nerve [34] and it has been questioned that cholinergic and adrenergic nerve profiles can be identified by the morphological types of synaptic vesicles [35]. Thus, it seems necessary to further examine the autonomic nerves by immunoelectron microscopic studies using antibodies against the neurotransmitters or related enzymes to the metabolism of nervous system such as tyrosine hydroxylase and cholineacetyltransferase [34, 36].

Finally, as for relevance to lens regeneration, the present results disclosed the detailed structure of the iris muscle in the non-operated eyes before lentectomy. Thus, the behavior of the iris muscle

during lens regeneration is now under investigation, because it would be interesting to know whether the differentiated iris muscle cells situated at the dorsal marginal iris can be transformed into lens cells.

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